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12/11

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1) Takaku H, 1992, Int J Cancer, 51 (2): 244-9.
2) Miyazaki K, 1990, Cancer Res, 50(15): 4522-7.
3) Takaku H, 1993, Japanese J Cancer Research, 84(11): 1195-200?
Thanks
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272-00830

[CANCER RESEARCH 50, 4522–4527, August 1, 1990]

Potent Growth Inhibition of Human Tumor Cells in Culture by Arginine Deiminase Purified from a Culture Medium of a *Mycoplasma*-infected Cell Line[1]

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ABSTRACT

Two kinds of growth-inhibitory substances were found in culture of a Rous sarcoma virus-transformed rat liver cell line, RSV-BRL. The two substances were purified from the serum-free culture medium and identified as transforming growth factor β_1 and *Mycoplasma*-derived arginine deiminase (EC 3.5.3.6), respectively. The arginine deiminase was an acid-labile but dithiothreitol-resistant protein with a molecular weight of 45,000 and pI 4.7. Its K_m value for L-arginine was 0.3 mM, which is about 30 times lower than that of bovine liver arginase. It was stable and active under culture conditions. When added into cultures, the arginine deiminase inhibited the growth of various human cancer cell lines at a dose of 5 ng/ml or higher by depleting L-arginine in the culture media. This effective dose was about 1000 times lower than that of bovine liver arginase. These results suggested the possibility of chemotherapeutic use of arginine deiminase for human cancers.

INTRODUCTION

Growth of animal cells is regulated by a variety of environmental factors such as growth factors, growth inhibitors, hormones, and nutrients. During the last few years, various types of growth-inhibitory proteins have been found in tissue extracts, body fluids, and culture media, indicating that negative growth regulators may be involved in control of cell proliferation (reviewed in Ref. 1). These studies seem to be important not only in clarifying the growth control mechanism of animal cells but also in developing new antitumor drugs.

We have been investigating growth-inhibitory substances using the nonmalignant rat liver cell line BRL and Rous sarcoma virus-transformed BRL (RSV-BRL) as the indicator cells (2). It was found previously that sera from rats and mice contain a growth inhibitor that preferentially acts on BRL cells, whereas rabbit serum has a growth inhibitor that preferentially acts on RSV-BRL cells (3). The growth inhibitor for the malignant cells has been partially purified from rabbit serum (4). Furthermore, it was recently found that the nonmalignant and malignant indicator cells (BRL and RSV-BRL) themselves secrete growth inhibitors into culture media (2, 5).

In the present study we attempted to characterize the growth-inhibitory substances present in the culture medium of RSV-BRL cells and found that one of the growth-inhibitory substances was *Mycoplasma*-derived arginine deiminase. This enzyme potently inhibited the growth of both BRL and RSV-BRL cells by causing arginine deficiency in their culture media. L-Arginine is a critical nutrient for the cultures of most types of mammalian cells. A similar arginine-degrading enzyme, arginase, is well known to exert growth-inhibitory activity on various cultured cells (6-8). Arginase can retard the growth of some experimental tumors *in vivo* (9, 10). To test the possibility of the arginine deiminase as an antitumor drug, its growth-inhibitory activity on various human cancer cells was also examined *in vitro*.

MATERIALS AND METHODS

Cells and Culture. The nontumorigenic epithelial cell line BRL, which had been established from liver cells of a normal adult Buffalo rat by H. Coon (11), was previously transformed by Rous sarcoma virus (12). From the culture of the transformed BRL, named RSV-BRL, five tumorigenic clones (RSV-BRL1 to RSV-BRL5) were obtained. RSV-BRL1 was used in this study. rasNHN3T3 was established by transfecting Kirsten murine sarcoma virus DNA (a gift from Dr. M. Yutsudo, Osaka University) into NIH3T3 cells. The cell lines HLE, HSC-3, HSC-4, T98G, RPMI-8226, and VMRC were provided from the Japanese Cancer Research Resource Bank. CaSki and C4I were purchased from Dainippon Seiyaku, Osaka, Japan. NIH3T3 was a kind gift from the late Dr. T. Kakunaga, Osaka University; BRL and SCC were from Dr. K. Nishikawa, Kanazawa Medical University; YH-1 and B-32 were from Dr. S. Gotoh, University of Occupational and Environmental Health; and A549 and KB were from Dr. N. Miwa, Hiroshima Prefecture University.

These cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The basal medium (DME/F12)[6] consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) and Ham's F-12 medium (GIBCO), which was supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, 1.2 mg/ml of $NaHCO_3$, 100 units/ml of penicillin G, and 0.1 mg/ml of streptomycin sulfate. The standard culture medium was 10% FCS plus DME/F12. Plastic culture wares were obtained from Becton Dickinson Labware (Oxnard, CA).

Assay of Growth Inhibitor Activity. The activity of growth inhibitors was routinely assayed with BRL as the indicator cells as described previously (3). In some experiments, RSV-BRL and other cell lines were used as the indicator cells. Unless otherwise noted, the indicator cells (2500/well) were seeded in duplicate on 24-well plates containing 0.5 ml/well of 10% FCS plus DME/F12 and incubated for 2–4 h to allow cell attachment. The cultures were then added with a small volume (5 to 25 μl) of test samples and further incubated. After 4 days in culture, the grown cells were counted with an automatic cell counter (Sysmex microcell counter CC-108; Kakogawa). In most cases, test samples were previously dialyzed against PBS (Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline) and sterilized by filtration. In control cultures, which were added with the same volume of PBS, the number of BRL cells increased 100- to 150-fold during the incubation. The ratio (×100) of the number of cells in a test culture to the averaged number of cells in control cultures was expressed as "relative cell number."

Assay of Arginine Deiminase Activity. The reaction mixture for the assay of arginine deiminase (EC 3.5.3.6) activity contained 10 mM L-arginine, 0.1 M potassium phosphate (pH 6.5), and 0.1 ml of an enzyme

Received 12/4/89; revised 4/10/90.

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[1] This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

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[6] The abbreviations used are: DME/F12, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium; GI-I and -II, growth inhibitor I and II, respectively; PBS, Dulbecco's phosphate-buffered saline (Ca^{2+} and Mg^{2+} free); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TGF-β, transforming growth factor β; 10% FCS plus DME/F12, DME/F12 supplemented with 10% fetal calf serum (GIBCO); HPLC, high-performance liquid chromatography.

solution in a final volume of 0.5 ml. The mixture was incubated at 37°C for 3 h unless otherwise indicated, and the enzyme reaction was terminated by adding 0.25 ml of a mixture of H_2SO_4 and H_3PO_4 (1:3, v/v). The citrulline formed during the incubation was determined with diacetyl monooxime according to the method of Oginsky (13).

Preparation of Conditioned Medium. RSV-BRL cells were grown to confluence in 10% FCS plus DME/F12. The cultures were then rinsed twice with PBS and incubated in serum-free DME/F12 overnight. The media were discarded and replaced with fresh serum-free DME/F12, and the cultures were continued. The serum-free conditioned media were harvested three times a week and clarified by sequential centrifugation at 800 × *g* for 15 min and at 20,000 × *g* for 30 min. The protein present in the clarified conditioned media was precipitated by 80% saturation of ammonium sulfate and collected by centrifugation at 20,000 × *g* for 30 min. The protein precipitates were dissolved in and dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl for the purification of growth inhibitors.

Column Chromatographies. Molecular sieve chromatography was carried out on a Cellulofine GCL2000 (Chisso Co., Ltd., Tokyo, Japan) column (2.6 x 98 cm), previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The concentrated conditioned medium of RSV-BRL cells was applied to the column and eluted with the same buffer at a flow rate of about 30 ml/h.

Heparin affinity chromatography was carried out on a heparin-agarose column (1.5 x 8.5 cm; Bio-Rad Laboratories, Richmond, CA), preequilibrated with 10 mM Tris-HCl buffer (pH 7.5). The GI-II pool from the molecular sieve chromatography was dialyzed against the same buffer and applied to the column. The charged column was washed with the buffer and eluted with a linear gradient of NaCl from 0 to 0.1 M in 400 ml of 10 mM Tris-HCl (pH 7.5) at a flow rate of 16 ml/h.

Cibacron Blue affinity chromatography was carried out on a Blue-Cellulofine column (1.0 x 5.0 cm; Chisso Co.) preequilibrated with 10 mM Tris-HCl buffer (pH 7.5). The GI-II pool obtained by heparin-agarose chromatography was applied to the column at a flow rate of 14 ml/h, and the adsorbed material was eluted with the buffer supplemented with 2 M NaCl and 6 M urea.

Hydrogen-bond chromatography was carried out on a Sepharose CL-6B column (1.0 x 2.5 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden), preequilibrated with 25 mM Tris-HCl (pH 7.5) containing 2 M ammonium sulfate, according to the method of Fujita *et al.* (14). The GI-II pool obtained by Blue-Cellulofine chromatography was supplemented with solid ammonium sulfate to 2 M and applied to the Sepharose column. The charged column was washed with the equilibration buffer and eluted with a linear gradient of ammonium sulfate from 2 M to 0 M in 140 ml of 25 mM Tris-HCl (pH 7.5) at a flow rate of 14 ml/h.

SDS-PAGE. SDS-PAGE was carried out on 10% polyacrylamide slab gels (90 mm long, 90 mm wide, 1 mm thick) with a Bio-Rad electrophoresis apparatus by the method of Laemmli (15). The molecular weight markers used are rabbit muscle phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,200), hen egg albumin (M_r 42,700), bovine carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 20,100), and hen egg lysozyme (M_r 14,300). After electrophoresis, the gels were stained with a "Wako" silver staining kit (Wako Chemicals, Osaka, Japan).

Isoelectric Electrophoresis. Sucrose gradient isoelectric electrophoresis was carried out in a 110-ml vertical column containing 1% (w/v) Ampholine-carrier ampholytes (pH 4–6/pH 3.5–10 = 2/8) (Pharmacia LKB Biotechnology) at 1°C, according to the method of Vesterberg and Svensson (16). After electrophoresis at 600 V for 40 h, the column content was collected in 1-ml fractions. The pH of each fraction was measured at 1°C.

Protein Microsequencing. About 30 μg of the GI-II protein purified by reverse-phase HPLC column were lyophilized and used for the structural analysis. The NH_2-terminal amino acid sequence was analyzed with an Applied Biosystems gas phase protein sequencer by courtesy of Dr. S. Tomasawa, Institute for Protein Research, Osaka University.

Determination of Protein Concentrations. Protein concentrations were determined by the dye method with a Bio-Rad Protein Assay Kit, using bovine serum albumin as the standard. Protein contents of purified growth inhibitors were estimated from the intensities of silver-stained protein bands on SDS-PAGE using the same standard protein as above.

Detection and Elimination of *Mycoplasma*. *Mycoplasma* contamination was tested with the bisbenzimidazole fluorochrome Hoechst 33258. Contaminating *Mycoplasma* was eliminated by treating the host cells twice with 1 μg/ml of an antibiotic, MC-210 (Dainippon Seiyaku) for 7 days.

RESULTS

Growth-inhibitory Substances Present in Conditioned Medium of RSV-BRL Cells. RSV-BRL cells were grown in serum-containing medium to reach confluency and then incubated in serum-free basal medium (DME/F12) for 2 days. The culture medium was collected and concentrated by ammonium sulfate precipitation. When the concentrated conditioned medium was added into cultures of BRL and RSV-BRL cells, their growth was inhibited dose dependently. To characterize the growth-inhibitory factors present in the conditioned medium, the conditioned medium was fractionated by molecular sieve chromatography. The resultant fractions were dialyzed against PBS and assayed for growth-inhibitory activity with BRL and RSV-BRL cells (Fig. 1*A*). The growth-inhibitory activity was separated into a major peak at an apparent molecular weight of 45,000 (fractions 52–59) and a minor peak at the void volume (fractions 32–34). Two additional activities were detected as shoulders in molecular weight ranges of 300,000–700,000 (fractions 42–50) and 10,000–40,000 (fractions 60–68). The large and small peak fractions respectively inhibited the growth of BRL and RSV-BRL cells to similar extents, while the two shoulder fractions were more inhibitory for BRL than RSV-BRL cells.

When small portions of the column fractions were incubated

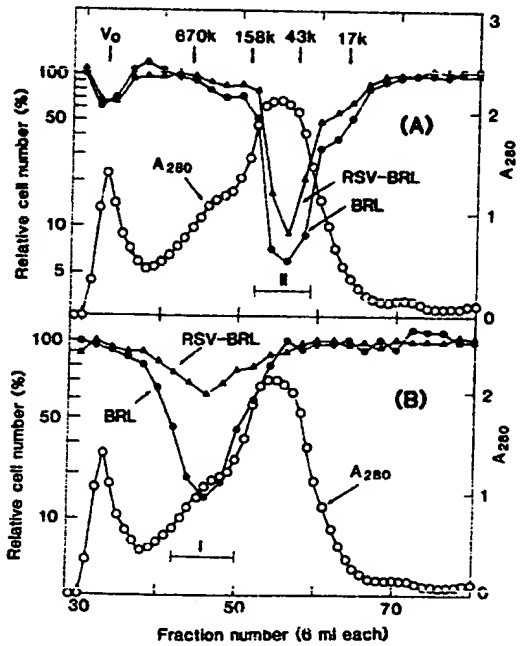

Fig. 1. Molecular sieve chromatography of concentrated conditioned medium of RSV-BRL cells on a Cellulofine GCL-2000 column. ●, relative number of BRL cells; ▲, relative number of RSV-BRL cells; ○, A_{280}. The elution positions of thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 43,000), myoglobin (M_r 17,000), and vitamin B_{12} (M_r 1,350) are indicated by *arrows*. (*A*) Growth-inhibitory activity was assayed at a dose of 100 μl/35-mm dish (total, 2 ml) after dialysis against PBS at 4°C overnight. (*B*) Growth-inhibitory activity was assayed at a dose of 5 μl/dish after dialysis against 1 M acetic acid (pH 2.3) at 4°C overnight. Fractions 42–50 (GI-I) and fractions 52–59 (GI-II) were respectively pooled and used for further purification. Other experimental conditions are given in the text.

with 1 M acetic acid and then assayed, the growth-inhibitory activity at M_r 300,000–700,000 increased about 100-fold to be the major activity, indicating that this growth inhibitor had been secreted as a latent or less active form into the culture medium (Fig. 1*B*). The major growth inhibitors in fractions 42–50 and 52–59 were tentatively designated as GI-I and GI-II, respectively.

GI-I present in fractions 42–50 was extracted with 1 M acetic acid and further purified by preparative SDS-PAGE under nonreducing conditions and then by isoelectric electrophoresis on a thin-layer polyacrylamide gel. When analyzed by SDS-PAGE, the purified inhibitor showed a homogeneous band with a molecular weight of 25,000 under nonreducing conditions or 13,000 under reducing conditions (data not shown). Its pI was about 9.2 as determined by polyacrylamide gel isoelectric electrophoresis. About 11 μg of GI-I were obtained from 6 liters of the conditioned medium. Analysis of the partial NH_2-terminal amino acid sequence has shown that GI-I must be TGF-β_1.

Purification of GI-II. The GI-II fractions obtained by the molecular sieve chromatography (fractions 52–59 in Fig. 1*A*) were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5), and subjected to affinity chromatography on a heparin-agarose column. The growth-inhibitory activity was weakly adsorbed to the column and eluted at about 0.06 M NaCl. The active fractions from the heparin-agarose column were pooled and then applied to a Cibacron blue-conjugated column (Blue-Cellulofine column). GI-II activity passed through the column without adsorption, although about 70% of the total protein was adsorbed to the column (data not shown). The nonadsorbed fractions were pooled, added with solid ammonium sulfate to make a final concentration of 2 M and then subjected to hydrogen bond chromatography on a Sepharose CL-6B column (Fig. 2). GI-II was adsorbed to the column at 2 M ammonium sulfate and eluted from the column by decreasing the ammonium sulfate concentration to about 1.4 M. The active fractions were pooled, dialyzed against 10 mM Tris-HCl (pH 7.5), and subjected to isoelectric electrophoresis in a sucrose gradient column (Fig. 3). The growth inhibitor was focused at pH 4.7. GI-II thus purified was analyzed by SDS-PAGE under nonreducing conditions (Fig. 4). The GI-II preparation showed a nearly homogeneous band with a molecular weight of 45,000. The electrophoretic mobility of the M_r 45,000 protein was hardly affected by treatment with 2-mercaptoethanol, showing that it was a single-chain peptide. In this purification procedure about 1 μg of GI-II was purified from 6 liters of the conditioned medium

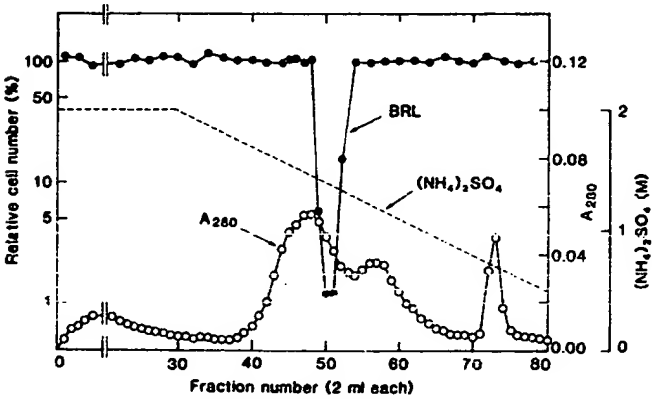

Fig. 2. Hydrogen bond chromatography on Sepharose CL-6B column of GI-II fraction obtained by Blue Cellulofine chromatography. Small portions of the column fractions were dialyzed against PBS and tested for growth-inhibitory activity at a dose of 10 μl/well. ○, A_{280}; ●, relative number of BRL cells; - - - -, ammonium sulfate concentration. Fractions 50 and 51 were pooled and used for further purification of GI-II. Other experimental conditions are given in the text.

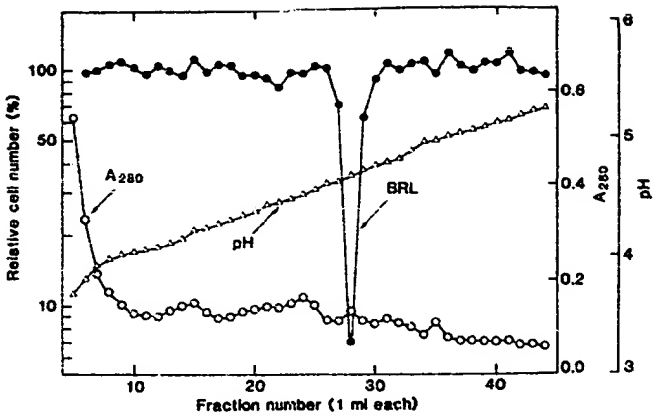

Fig. 3. Sucrose gradient isoelectric electrophoresis of GI-II pool obtained by hydrogen bond chromatography. After electrophoresis, the column fractions were dialyzed against PBS and tested for growth-inhibitory activity at a dose of 5 μl/well. ○, A_{280}; ●, relative number of BRL cells; △, pH. Fraction 28 was used as purified GI-II.

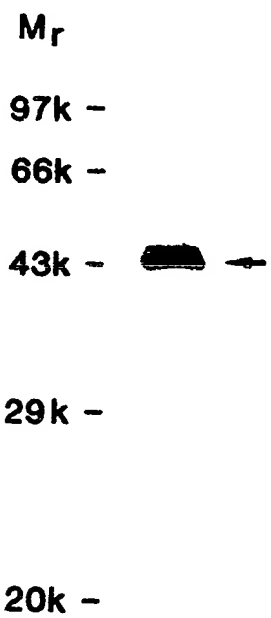

Fig. 4. SDS-PAGE of purified GI-II under nonreducing conditions. Protein bands were detected by the silver staining. *k*, molecular weight in thousands.

Ser-Val-Phe-Asp-Ser-Lys-Phe-Lys-Gly-Ile-
His-Val-Tyr-Ser-Glu-Ile-Gly-Gly-

Fig. 5. NH_2-terminal amino acid sequence of purified GI-II.

with an activity yield of 8% and with a 1200-fold enrichment.

In another experiment, 100 liters of the serum-free conditioned medium of RSV-BRL cells were used as the starting material to prepare the GI-II protein for structural analysis. In this purification the isoelectric electrophoresis was replaced with anion-exchange chromatography on a DEAE-Sepharose column. The active GI-II fraction obtained from the chromatography was finally subjected to reverse-phase HPLC on an Altex Ultrapore C3 column. Although the acidic conditions of the reverse-phase HPLC inactivated GI-II, about 30 μg of the GI-II M_r 45,000 protein were purified to homogeneity. The 18 NH_2-terminal amino acids of the M_r 45,000 protein were determined with an automatic gas-phase protein sequencer (Fig. 5). A computer search indicated that the NH_2-terminal sequence had no significant homology to any known protein in the National Biomedical Research Foundation protein data

bank (Release No. 18) and in the Swiss-Pro data bank (Release No. 9.0).

Characterization of GI-II. The growth-inhibitory activities of the purified GI-I (TGF-β_1) and GI-II (fraction 28 in Fig. 3) on BRL and RSV-BRL are shown in Fig. 6. GI-I inhibited the growth of BRL much more potently than RSV-BRL. In most cases the extent of the maximal growth inhibition ranged from 75 to 85% for BRL and from 20 to 40% for RSV-BRL. The dose required for 50% growth inhibition was about 1 ng/ml for BRL. On the other hand, GI-II completely inhibited the growth of both BRL and RSV-BRL cells, showing a sigmoidal dose-response curve. The dose required for 50% growth inhibition was about 4 ng/ml for both kinds of cells. The effect of GI-II on these cells appeared cytostatic during the initial 3 days in incubation, and the majority of the cells were alive and could grow normally if the medium was replaced with a fresh one without GI-II. However, prolonged incubation with an excess amount of GI-II increased the number of dead cells. The unique dose-response curve of GI-II suggested some special growth-inhibitory mechanism of this protein.

There are some reports indicating that arginase exerts a growth-inhibitory effect on cultured mammalian cells by converting L-arginine present in the culture media to ornithine (6–8). In addition, *Mycoplasma*-derived arginine deiminase, which converts L-arginine to citrulline, has been suggested to show a similar growth-inhibitory activity (17, 18). Therefore, the effect of GI-II on amino acid composition was examined under the conditions for the assay of growth-inhibitory activity (Table 1). When GI-II was incubated with BRL cells at 37°C, the arginine content in the medium gradually decreased and became undetectable after 4 days. The decrease of arginine content was accompanied with a reciprocal increase of citrulline content, indicating that the culture contained arginine deiminase. The changes for other amino acids, if any, were far less than that for arginine. As expected, the addition of excess amounts of L-arginine into the culture of BRL cells reversed the growth inhibition caused by GI-II in a dose-dependent manner (Fig. 7). This indicated that the growth inhibition by GI-II was due to the depletion of L-arginine in culture medium. The addition of 1 mM citrulline into culture medium gave no growth-inhibitory effect on the indicator cells (data not shown).

The incubation of GI-II with L-arginine in a cell-free reaction mixture directly proved that this protein had arginine deiminase activity (Table 2). The K_m value of the arginine deiminase for L-arginine was determined to be about 0.3 mM (data not shown). The stability of the arginine deiminase under various conditions is summarized in Table 3. This enzyme is stable at neutral pH but unstable in acid. About 50% of the activity remained after

Table 1 *Effect of purified GI-II on amino acid composition in culture medium*

BRL cells were cultured in 10% FCS plus DME/F12 with or without GI-II. After the incubation for the indicated lengths of time, the culture media were collected and clarified by sequential centrifugations at 800 × *g* for 15 min and then at 20,000 × *g* for 30 min. Proteins were precipitated by adding 0.5 ml of 55% (w/v) trichloroacetic acid to 1 ml of the media, followed by centrifugation. Amino acids in the resultant supernatants were adsorbed to an Amberlite IR-120 column (0.5 × 5 cm) and then eluted with 3 M NH_4OH. The eluted amino acid samples were lyophilized and subjected to a PICO-TAG amino acid analyzer (Millipore, Milford, MA). Cit and Orn indicate citrulline and ornithine, respectively.

	Concentration in medium (mM) at incubation times					
	+ GI-II (5 ng/ml)					None,
Amino acid	0	1 day	2 days	3 days	4 days	4 days
Asp	0.052	0.047	0.045	0.040	0.042	0.038
Glu	0.178	0.162	0.167	0.163	0.192	0.175
Ser + Asn	0.231	0.209	0.204	0.185	0.194	0.179
Gly + Gln	2.101	1.834	1.712	1.523	1.500	1.438
His	0.123	0.106	0.107	0.100	0.103	0.107
Arg	0.562	0.328	0.149	0.050	0.000	0.355
Thr	0.366	0.328	0.325	0.296	0.330	0.314
Ala	0.154	0.152	0.175	0.194	0.285	0.296
Pro	0.148	0.140	0.136	0.125	0.141	0.124
Tyr	0.172	0.158	0.155	0.146	0.155	0.147
Val	0.392	0.359	0.361	0.335	0.344	0.323
Met	0.103	0.093	0.088	0.085	0.083	0.075
Cys	0.090	0.049	0.065	0.073	0.060	0.055
Ile	0.349	0.318	0.320	0.286	0.279	0.259
Leu	0.379	0.348	0.347	0.315	0.314	0.285
Phe	0.183	0.169	0.172	0.162	0.174	0.162
Trp + Orn	0.100	0.136	0.190	0.162	0.174	0.276
Lys	0.430	0.393	0.396	0.370	0.397	0.371
Cit	0.016	0.135	0.298	0.398	0.467	0.013

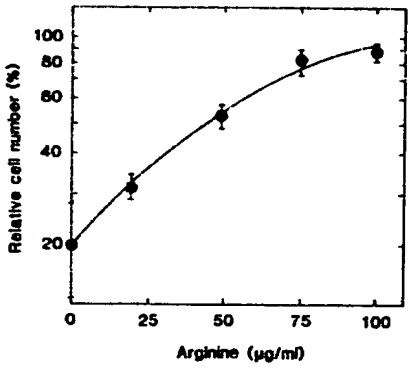

Fig. 7. Effect of addition of extra L-arginine on growth-inhibitory activity of purified GI-II on BRL cells. The indicated amounts of L-arginine were added into 0.5 ml of culture medium containing BRL cells and GI-II (2.5 ng). Each *point* represents the average for duplicate wells; *bars*, range of values.

Table 2 *Arginine deiminase activity of purified GI-II*

Concentration (ng/ml)	Citrulline formed (nmol/24 h)
0	0
10	260
20	520

Table 3 *Stability of GI-II (arginine deiminase)*

Treatment	Arginine deiminase activity (% of control)
Control (PBS, pH 7.2)	(100)
4°C for 3 wk	94
37°C for 24 h	51
60°C for 30 min	10
100°C for 5 min	0
0.1 M acetic acid (pH 2.8) (4°C for 24 h)[a]	11
PBS + 50 mM dithiothreitol (4°C for 24 h)	97
PBS + 6 M urea (4°C for 24 h)	58

[a] GI-II was dialyzed against 0.1 M acetic acid at 4°C for 24 h.

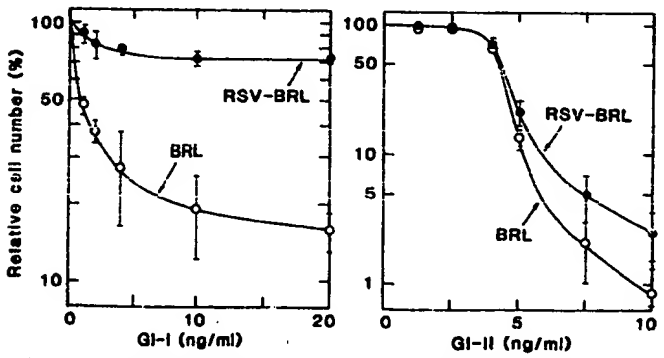

Fig. 6. Effects of various concentrations of purified GI-I (*left*) and GI-II (*right*) on growth of BRL (○) and RSV-BRL (●) cells. Each *point* represents the average for duplicate wells; *bars*, range of values.

incubation at 37°C for 24 h, although its activity was mostly lost after incubation at 60°C for 30 min or at 100°C for 5 min. The arginine deiminase was resistant to 50 mM dithiothreitol but partially inactivated by 6 M urea.

The test for *Mycoplasma* infection revealed the presence of *Mycoplasma* in the culture of RSV-BRL cells. When the contaminating *Mycoplasma* organisms were removed from the culture of RSV-BRL cells by the use of an antibiotic MC210, the growth-inhibitory activity in the conditioned medium markedly decreased and the arginine deiminase activity disappeared (Table 4). These results demonstrated that GI-II is *Mycoplasma*-derived arginine deiminase.

Growth-inhibitory Activity of GI-II (Arginine Deiminase) on Various Cell Lines. The results described above clearly show that the arginine deiminase derived from mycoplasmas contaminating RSV-BRL cells inhibits cell growth by consuming L-arginine in culture medium. To compare its growth-inhibitory activity with that of a similar arginine-degrading enzyme, arginase, commercial bovine liver arginase with a specific activity of 150–250 units/mg protein (Sigma Chemical Company, St. Louis, MO) was tested for growth-inhibitory activity on BRL cells. The arginase inhibited the growth of BRL at doses higher than 5 μg/ml, about 1000 times the effective doses of the arginine deiminase (data not shown). Bovine liver arginase has a K_m of 10.5 mM, about 30 times that of the arginine deiminase, and an optimum pH at 9.3 (19). The marked difference in their effective doses appears to come largely from the difference in their K_m values.

The *in vitro* growth-inhibitory activity of GI-II was tested with 3 pairs of nontransformed and transformed cell lines and with 11 kinds of human cancer cell lines (Table 5). GI-II nonspecifically inhibited the growth of the nontransformed and transformed BRL and NIH3T3, but it was more inhibitory for SV40-transformed human fibroblast line than its nontransformed counterpart. GI-II more or less inhibited the growth of all human cancer cell lines tested. Among them, HLE (hepatoma), CaSki (cervix squamous cell carcinoma), and VMRC (melanoma) were especially sensitive to the arginine-degrading enzyme.

Table 4 *Growth-inhibitory and arginine deiminase activities of concentrated conditioned media obtained from Mycoplasma-free and Mycoplasma-containing RSV-BRL cultures*

Serum-free conditioned media were collected from *Mycoplasma*-free and -containing RSV-BRL cultures and concentrated 30-fold by ammonium sulfate precipitation. Growth-inhibitory activity on BRL cells was assayed on 24-well plates containing 0.5 ml/well of 10% FCS plus DME/F12.

Dose (μl)	Relative no. of BRL cells (% of control)	Citrulline formed (nmol/24 h)
Mycoplasma-free		
25	75	0
50	52	0
Mycoplasma-containing		
25	46	55
50	2	127

Table 5 *Effect of GI-II (arginine deiminase) on growth of various cell lines*

Each cell line, except for BRL and RSV-BRL, was seeded on 24-well plates at a density of 1×10^4 cells/well containing 0.5 ml of 10% FCS plus DME/F12, and incubated with two different concentrations of GI-II. BRL and RSV-BRL were seeded at a density of 2500 cells/well. Other experimental conditions are given in "Materials and Methods."

Cell line (type)	Relative cell no.[a] (% of control) 5 ng/ml	10 ng/ml
Nontransformed and transformed		
BRL (rat liver epithelial cell)	10.3 ± 2.4	5.2 ± 0.4
RSV-BRL (RSV-transformed BRL)	14.6 ± 8.7	7.8 ± 3.2
NIH3T3 (mouse fibroblast)	46.2 ± 8.2	27.2 ± 1.7
rasNIH3T3 (*ras*-transformed NIH3T3)	41.7 ± 2.6	21.3 ± 0.4
YH-1 (human skin fibroblast)	55.1 ± 4.6	41.8 ± 1.3
B-32 (SV40-transformed YH-1)	20.1 ± 1.6	10.3 ± 1.0
Human cancer		
HLE (hepatoma)	16.7 ± 1.3	6.7 ± 0.5
HSC-3 (tongue squamous carcinoma)	69.0 ± 9.0	60.4 ± 6.1
HSC-4 (tongue squamous carcinoma)[b]	57.4 ± 4.6	41.8 ± 1.3
CaSki (cervix squamous carcinoma)	31.6 ± 3.1	22.9 ± 1.3
C41 (cervix squamous carcinoma)	63.6 ± 10.1	60.6 ± 12.3
A549 (lung adenocarcinoma)	42.3 ± 6.9	35.8 ± 3.0
SCC (colon adenocarcinoma)	63.5 ± 4.9	60.1 ± 13.3
KB (nose adenocarcinoma)	57.1 ± 7.2	46.9 ± 8.0
T98G (glioblastoma)[b]	63.8 ± 1.4	43.1 ± 2.5
RPMI-8226 (myeloma)	62.9 ± 9.4	58.1 ± 8.3
VMRC (melanoma)	41.7 ± 9.5	23.4 ± 3.1

[a] The values represent mean ± SD obtained from four wells. The averaged cell number per well ($\times 10^{-4}$) in control cultures after 4 days was 38.5 (BRL), 37.8 (RSV-BRL), 6.94 (NIH3T3), 23.4 (rasNIH3T3), 21.1 (B-32), 15.1 (HLE), 8.18 (HSC-3), 15.7 (HSC-4), 5.64 (CaSki), 5.32 (C41), 11.1 (A549), 8.14 (SCC), 28.8 (KB), 9.22 (T98G), 19.6 (RPMI), and 5.74 (VMRC).
[b] Mycoplasmas were detected.

DISCUSSION

In the present study two kinds of growth-inhibitory substances (GI-I and GI-II) were purified from the conditioned medium of RSV-BRL cells and identified as TGF-β_1 and arginine deiminase, respectively.

TGF-β is a family of structurally and functionally related growth regulators (reviewed in Refs. 1, 20, and 21). TGF-β_1 is secreted from many types of cultured cells in a latent form (22, 23). RSV-BRL also secreted TGF-β_1 as a latent protein complex with molecular weights of 300,000 to 700,000 into culture medium. The protein complex showed only a slight growth-inhibitory activity, but the treatment with acid increased its activity about 100-fold by liberating the active M_r 25,000 TGF-β_1 from the complex. The active TGF-β_1 potently inhibited the growth of BRL cells but hardly that of RSV-BRL cells, from which TGF-β_1 had been secreted. We have previously reported similar selective growth inhibition of BRL cells by growth inhibitors prepared from rat serum and human platelets (3, 24). There is no doubt that TGF-β plays fundamental roles in control of cell growth and other various cellular functions. However, it seems hopeless to use TGF-β as an antitumor agent because of its relatively low activity on malignant cells.

Arginine deiminase (EC 3.5.3.6) is abundant in microorganisms such as *Mycoplasma*, bacteria, and yeast. The arginine deiminases purified from these sources are composed of two identical subunits with a molecular weight of approximately 50,000 (25, 26). The arginine deiminase (GI-II) found in the conditioned medium of RSV-BRL cells was derived from the *Mycoplasma* contaminating the malignant rat liver cells. The arginine deiminase purified from the culture of RSV-BRL cells appeared to be a single peptide (M_r 43,000), but this should be further confirmed because abnormal elution of proteins is often observed in molecular sieve chromatography.

Mycoplasma infection is one of the most important problems in studies on cell culture. Mycoplasmas often affect the metabolism and function of the host cells. It has been reported that the infection of arginine-utilizing mycoplasmas or the addition of their extracts inhibits the growth of mammalian cells in culture (17, 18). These previous studies suggested that arginine deiminase might be the growth-inhibitory principle present in mycoplasmas inasmuch as the growth inhibition by mycoplasmas or their extracts was prevented by adding an excess amount of L-arginine into culture medium. However, growth-inhibitory activity of purified arginine deiminase has not been reported before. The arginine deiminase purified in this study inhibited

the growth of various murine and human cell lines as little as 5 ng/ml.

The use of amino acid-degrading enzymes as antitumor agents is one of the approaches for the treatment of human cancer. The most representative example is asparaginase (27). It has been successfully used for the treatment of lymphoblastic leukemia, leukemic lymphosarcoma, and lymphosarcoma. Arginase is another enzyme that exerts growth-inhibitory or cytotoxic effect on various cultured cells (6–8). This enzyme inhibits cell growth by the same mechanism as arginine deiminase. Bach and Swaine (9) showed that arginase could retard the growth of Walker carcinoma *in vivo*. Savoca *et al.* (10) reported that arginase covalently bound with polyethylene glycol, but not native enzyme, effectively extended the survival times of mice given injections of Taper liver tumor cells. The effectiveness of such amino acid-degrading enzymes as antitumor agents depends on their enzymatic properties (K_m, optimum pH, specific activity, stability at neutral pH), selectivity on tumor cells, stability in blood circulation, and immunogenicity. The attachment of polyethylene glycol to proteins can reduce their immunogenicity in animals and increase their stability to various hydrolytic enzymes, resulting in the increase of their blood circulatory lives (10, 28).

Our study demonstrated that the *in vitro* growth-inhibitory dose of the arginine deiminase (GI-II) was about 1000 times lower than that of bovine liver arginase. It is very likely that the great difference in their minimum effective doses derives largely from the difference in their K_m values for L-arginine: 0.3 mM with the arginine deiminase and 10.5 mM with the arginase. As shown in Table 5, the growth-inhibitory effect of the arginine deiminase appears specific for neither transformed nor tumorous cells. Although it was more or less growth-inhibitory for all of 11 human cancer cell lines tested, three of them, HLE, CaSki, and VMRC, were specially sensitive to this enzyme. This suggests the chemotherapeutic value of arginine deiminase for some specific kinds of human cancers. We did not identify the type of *Mycoplasma* which had contaminated RSV-BRL cells. Preliminary studies have shown that the arginine deiminase purified from *Mycoplasma arginini* has a similar growth-inhibitory activity. The *in vitro* and *in vivo* antitumor activities of various kinds of arginine deiminases are currently under investigation.

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272-00830

Int. J. Cancer: 51, 244–249 (1992)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

IN VIVO ANTI-TUMOR ACTIVITY OF ARGININE DEIMINASE PURIFIED FROM *MYCOPLASMA ARGININI*

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Arginine deiminase (EC 3.5.3.6) was purified to homogeneity from the cell extract of *Mycoplasma arginini* by molecular-sieve, anion-exchange and arginine-affinity chromatographies. The purified enzyme was composed of 2 identical sub-units with a molecular weight of 45.000 and had a pI of 4.7. Its V_{max} value and K_m value for L-arginine were estimated to be 50 units/mg protein and 0.2 mM, respectively. It exerted maximal enzyme activity at pH 6.0–7.5 and at 50°C. The arginine deiminase was stable at neutral pH. When injected i.v. into mice, the half-life of the arginine deiminase in blood was about 4 hr. In culture, the enzyme strongly inhibited the growth of 6 kinds of mouse tumor cell lines by depleting L-arginine in the culture media. When the *in vivo* growth-inhibitory activity of arginine deiminase was tested for the 6 tumor cell lines, i.p. administration of the purified enzyme effectively prolonged the survival time of the mice injected with all kinds of the tumor cell lines. Especially, the *in vivo* growth of a hepatoma cell line, MH134, was completely prevented by the daily administration at a dose of 0.2 mg/mouse for 14 days. These results raise the possibility of the use of the arginine deiminase derived from *Mycoplasma arginini* as a new anti-tumor drug.

The use of amino-acid-degrading enzymes is one of the ways to treat human cancer. So far only L-asparaginase (EC 3.5.1.1) has been successfully used for the treatment of human cancer. However, its use is limited to a few kinds of tumors, such as lymphoblastic leukemia and lymphosarcoma (Robert *et al.,* 1966; Broome *et al.,* 1968). Arginase (EC 3.5.3.1) is known to inhibit the growth of various cultured mammalian cells by consuming L-arginine in the culture media, but it has not been applied to the treatment of human cancer because of its poor anti-tumor activity *in vivo* (Bach and Swaine, 1965; Terayama *et al.,* 1982). Arginine deiminase (EC 3.5.3.6), which catalyzes the hydrolysis of L-arginine into L-citrulline and ammonia, seems to be a possible anti-tumor enzyme. L-arginine is an essential nutrient for most mammalian cells and a major energy source in various non-glycolytic arginine-utilizing Mycoplasma. In the Mycoplasma, the amino acid is catabolized to produce adenosine 5′-triphosphate (ATP) by the arginine dihydrolase pathway consisting of 3 enzymes, arginine deiminase, ornithine transcarbamylase and carbamate kinase (Schimke *et al.,* 1966; Fenske and Kenny, 1976). Arginine deiminase also exists in other types of micro-organisms such as *Tetrahymena pyriformis* (Hill and Chambers, 1967), *Streptococcus faecalis, Pseudomonas putida* and baker's yeast (Cunin *et al.,* 1986; Robert *et al.,* 1987).

It is known that the infection of arginine-utilizing Mycoplasma or the addition of their cell extracts inhibits the growth of cultured mammalian cells by depleting L-arginine in the culture media (Kenny and Pollock, 1962; Kraemer, 1964; Gill and Pan, 1970; Miller *et al.,* 1971; Sasaki *et al.,* 1984). Sugimura *et al.* (1990) have identified a lymphocyte-blastogenesis-inhibitory factor purified from the culture medium of a human histiocytic lymphoma cell line as arginine deiminase derived from *Mycoplasma arginini* contaminating the culture.

We previously found that arginine deiminase purified from culture medium of a Mycoplasma-infected rat cell line inhibited the growth of various human cancer cell lines at very low concentrations (5 ng/ml or higher) (Miyazaki *et al.,* 1990). These effective concentrations were about 100 times lower than those of bovine liver arginase. The marked difference between their effective concentrations was ascribed to the difference between their Km values for L-arginine. A similar growth-inhibitory effect was observed with the arginine deiminase derived from *M. arginini.* However, *in vivo* anti-tumor activity has not been reported with any kind of arginine deiminase. These facts prompted us to investigate the chemotherapeutic value of arginine deiminase for cancer treatment. Arginine deiminase has been purified and characterized from some strains of Mycoplasma (Weickmann and Fahrney, 1977; Kondo *et al.,* 1990; Ohno *et al.,* 1990). *M. arginini* is known to possess high activity of arginine deiminase (Sasaki *et al.,* 1984), but its enzymological properties have not been characterized. This paper reports the properties of arginine deiminase purified from *M. arginini* and its *in vivo* and *in vitro* anti-tumor activities for 6 kinds of mouse ascites tumor cell lines.

MATERIAL AND METHODS

Mouse tumor cell lines

Mouse tumor cell lines Colon 26 (colon carcinoma), Meth A (fibrosarcoma) and B16 (melanoma) were kind gifts from Dr. T. Tashiro, Japanese Foundation for Cancer Research, Tokyo; MH134 (hepatoma) was kindly donated by Dr. H. Taguchi, SRL Company, Tokyo; S-180 (sarcoma) and L1210 (leukemia) were purchased from Dainippon Seiyaku, Tokyo.

Assay of arginine deiminase activity

Arginine deiminase (EC 3.5.3.6) activity was assayed as described by Miyazaki *et al.* (1990). One milliliter of the reaction mixture, consisting of 10 mM L-arginine, 0.1 M potassium phosphate (pH 7.0) and an enzyme sample, was incubated at 37°C for 5 min, and the reaction was stopped by adding 1.0 ml of a 1:3 mixture (v/v) of conc. H_2SO_4 and conc. H_3PO_4. The amount of citrulline formed during incubation was determined by the formation of a colored reaction product with diacetyl mono-oxime according to the method of Oginsky (1957). One unit of enzyme activity was defined as the amount of enzyme which converted 1 μmol of L-arginine to L-citrulline per min in the assay conditions.

Purification of arginine deiminase from M. arginini

M. arginini (ATCC 23838) was provided from the Institute for Fermentation, Osaka. The Mycoplasma cells were cultured in 20 liters of Bacto PPLO broth w/o CV (Difco, Detroit, MI) (pH 7.0) containing 20% horse serum (Irvine Scientific, Santa Ana, CA), 2.5% yeast extract (Difco) and 1.0% L-arginine monohydrochloride. The cultured cells were collected by

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Abbreviations: Mr, molecular weight; PBS, Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Received: September 9, 1991 and in revised form December 18, 1991.

centrifugation at 15,000 *g* for 20 min, washed twice with PBS, suspended in 30 ml of 10 mM potassium phosphate buffer (pH 7.0), and sonicated at 20 kHz for 15 min in an ice bath. The sonicated cell suspension was centrifuged at 100,000 *g* for 60 min at 4°C. The Mycoplasma cell extract was subjected to molecular-sieve chromatography on a Cellulofine GCL-2000m (Chisso, Tokyo) column (2.6 × 98 cm), previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 M NaCl, at a flow rate of 24 ml/h. The active fractions obtained from the molecular-sieve column were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 7.0), and applied to anion-exchange chromatography on a DEAE-Toyopearl (Toso, Tokyo) column (2.6 × 10 cm), previously equilibrated with the phosphate buffer. The loaded column was washed with 150 ml of the same buffer and then eluted with a linear gradient of 0–0.5 M NaCl in 300 ml of the phosphate buffer at a flow rate of 60 ml/h. The arginine-deiminase-rich fractions obtained by the anion-exchange chromatography were pooled, dialyzed against 10 mM potassium phosphate buffer (pH 7.0), and applied to an arginine-Sepharose 4B (Pharmacia, Uppsala, Sweden) column (2.2 × 10 cm) equilibrated with the same phosphate buffer (pH 7.0). The column was washed with 100 ml of the same buffer and then eluted with a linear gradient of 0–1.0 M NaCl in 600 ml of phosphate buffer at a flow rate of 50 ml/hr. All purification procedures were carried out at 4°C.

Electrophoretic analyses

SDS-PAGE was performed on 10% polyacrylamide gels (90 mm long, 90 mm wide, 1 mm thick) by the method of Laemmli (1970). Polyacrylamide gel isoelectric focusing was performed on an Ampholine PAG plate (110 mm long, 245 mm wide, 1 mm thick) with a pH gradient of 3.5–9.5 (Pharmacia). The pH gradient formed on the gel was determined using a pI calibration kit (Pharmacia).

Analysis of amino acid in plasma

Purified arginine deiminase (0.1 mg/mouse) was injected into the tail vein of 3 normal BDF_1 mice. Blood samples were obtained periodically from the tail vein, and amino acids in the blood were modified by fluorescent reagent with NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) according to the method of Imai and Watanabe (1984). Modified arginine and citrulline were analyzed by reverse-phase HPLC on a μ-Bondapack C_{18} (Waters, Milford, MA) column (10 μm, 3.9 × 300 mm).

Assay of in vitro *growth-inhibitory activity*

The growth-inhibitory activity of arginine deiminase was assayed for 6 kinds of mouse tumor cell lines. The test cells were inoculated in triplicate on 24-well microplates containing 1.0 ml of MEM supplemented with 10% FCS at a density of 1×10^4 cells/well. The cultures were then added with 20 μl of arginine deiminase, which had been previously dialyzed against PBS and sterilized by filtration, to a final concentration of 2.5 to 100 ng/ml, and incubated at 37°C in a humidified 5% CO_2–95% air atmosphere. After 3 days in culture, the grown cells were counted with an automatic cell counter (Coulter, Hialeah, FL).

Assay of in vivo *growth-inhibitory activity*

The mouse strain used were the CDF_1 strain for L1210 cells and Colon26 cells, the BDF_1 strain for B16 cells, the BALB/c strain for MethA cells, the ICR strain for S-180 cells and the C3H/HeN strain for MH134 cells. For each experiment, 5 to 10 male mice of 7 weeks old were injected i.p. with 1×10^6 cells of a tumor line. The injected mice were randomly divided into control and test groups. The purified arginine deiminase, which had previously been dialyzed against PBS and sterilized by filtration, was injected i.p. into the mice of the test group once a day for 14 days from the next day of the tumor cell injection. The control mice were given PBS.

RESULTS

Purification of arginine deiminase

Two g wet weight of *M. arginini* cells was obtained from 2 liters of the culture medium, and used as the starting material for the purification of arginine deiminase. The cell extract of *M. arginini* was fractionated by molecular-sieve chromatography on a Cellulofine GCL-2000m column. The arginine deiminase activity was eluted in fractions corresponding to an apparent Mr of 100,000 (data not shown). These fractions were pooled, dialyzed, and subjected to anion-exchange chromatography on a DEAE-Toyopearl column (Toso). The enzyme was adsorbed to the column and eluted, forming a single activity peak at about 0.25 M NaCl (data not shown). The peak fractions were combined and finally applied to an arginine-Sepharose 4B column. In this affinity chromatography, the enzyme activity was adsorbed to the column and eluted at about 0.8 M NaCl, forming a single protein peak (Fig. 1). By the 3-step procedure, 14 mg of pure arginine deiminase was obtained from the 2-liter culture of *M. arginini* with an activity yield of 38% and with 8.2-fold enrichment.

Properties of arginine deiminase

The purified arginine deiminase gave a single band with Mr 45,000 on SDS-PAGE in non-reducing conditions (Fig. 2*a*). Its electrophoretic mobility was not affected by treatment with 2-mercaptoethanol, showing that it contained no intermolecular disulfide bond (data not shown). The molecular weight of the enzyme in its native form was estimated to be about 90,000 as analyzed by molecular-sieve HPLC on a TSKgel $G3000SW_{XL}$ column (Fig. 3). These results suggested that the arginine deiminase was a dimeric protein consisting of 2 identical subunits.

When the purified enzyme was subjected to isoelectric focusing on a polyacrylamide slab gel, it formed a single band with pI 4.7, indicating that no isoelectric isoenzyme was contained (Fig. 2*b*).

The Vmax value and the Km value for L-arginine of the enzyme were determined to be 50 units/mg protein and 0.2 mM, respectively, by kinetic analysis with Lineweaver-Burk plots (Fig. 4). When the enzyme reaction was carried out in a pH range from 4.0 to 9.0, the maximal activity was attained at pH 6.0 to 7.5 (data not shown).

The enzyme was stable at neutral pH for more than one month at 4°C, but unstable at acidic pH. When incubated at 37°C for 24 hr, 50% to 60% of the enzyme activity remained (data not shown).

Plasma-circulating life of arginine deiminase was examined by injecting 0.1 mg of the purified enzyme into the tail vein of normal BDF_1 mice (Fig. 5). The enzyme activity decreased to 41% of the initial activity within 6 hr and to 17% within 24 hr. The half-life of the injected enzyme in mouse blood was estimated to be approximately 4 hr. The analysis of arginine level in the plasma of the arginine-deiminase-injected-mice showed that arginine was completely cleared from blood within 3 min after the enzyme injection, with a reciprocal increase of citrulline concentration (Table I). The plasma arginine level remained non-detectable for at least 3 days.

In vitro *growth-inhibitory activity*

The *in vitro* growth-inhibitory activity of the purified arginine deiminase on Meth A, Colon 26, B16, L1210, S-180 and MH134 is shown in Figure 6. The enzyme strongly inhibited the growth of all the mouse tumor cell lines showing sigmoidal dose-response curves. The concentration required for 50% growth inhibition (IC_{50}) ranged from 5 to 20 ng/ml for these tumor cells. When the enzyme was added into the cultures at

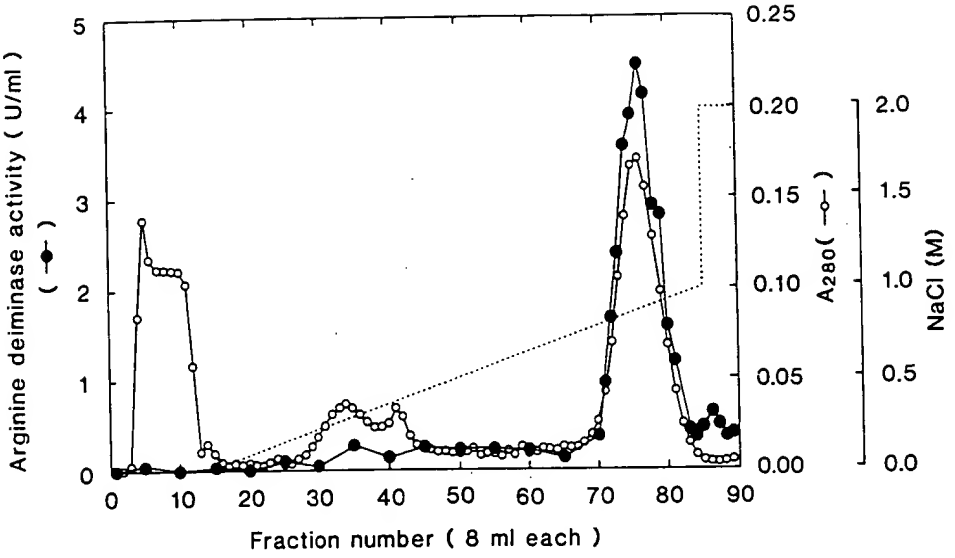

FIGURE 1 – Affinity chromatography on arginine-Sepharose 4B column of arginine deiminase fraction obtained by anion-exchange chromatography. ●, arginine deiminase activity; ○, A_{280}; ---, NaCl concentration. Fractions 70–80 were combined and used as purified arginine deiminase.

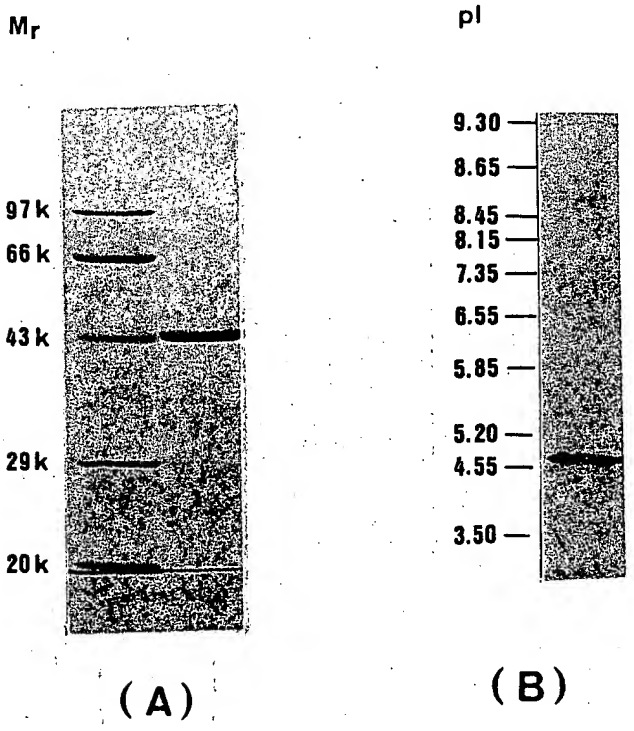

FIGURE 2 – SDS-PAGE under non-reducing conditions *(a)* and polyacrylamide gel isoelectric focusing *(b)* of purified arginine deiminase. Protein bands were stained with Coomassie brilliant blue R-250. *k*, molecular weight in thousands.

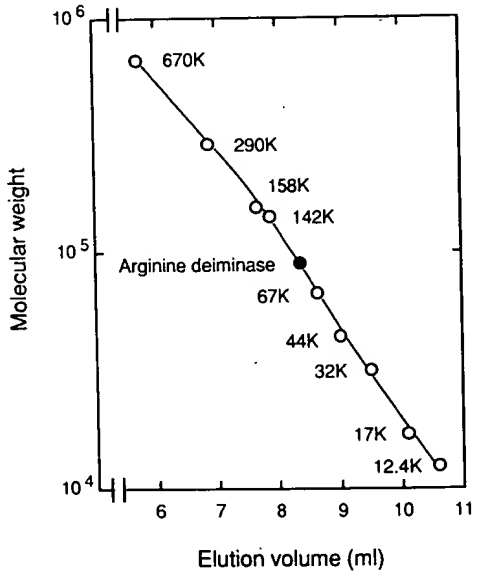

FIGURE 3 – Molecular-weight determination of arginine deiminase by molecular-sieve HPLC on TSK-G3000SW_{XL} column. The protein standards used are thyroglobulin (M_r 670,000), glutamate dehydrogenase (M_r 290,000), gamma globulin (M_r 158,000), lactate dehydrogenase (M_r 142,000), enolase (M_r 67,000), ovalbumin (M_r 44,000), adenylate kinase (M_r 32,000), myoglobin (M_r 17,000) and cytochrome c (M_r 12,400).

excess concentrations, Meth A, Colon 26, S-180 and MH134 cells were almost completely killed during the 3-day incubation, whereas in the cultures of B16 and L1210 a small proportion of cells remained alive. The incomplete growth inhibition in the latter group suggested that they contained cells resistant to the depletion of L-arginine in their cultures. During the first 1 or 2 days in culture, the growth-inhibitory effect of the arginine deiminase appeared cytostatic against all kinds of cell lines. These cells could grow normally if the medium was replaced by a fresh medium, either without arginine deiminase or with added (excess) L-arginine. L-citrulline and ammonia, the products of the enzymatic reaction, had no growth-inhibitory activity up to 2 mM (data not shown). These results indicated that growth inhibition by arginine deiminase was due to the depletion of L-arginine but not its reaction products.

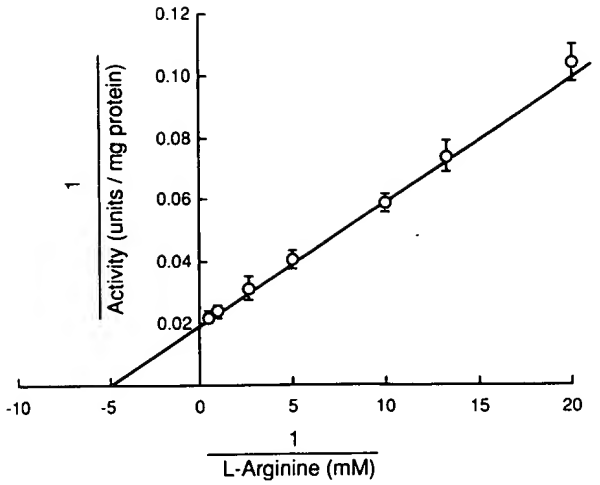

FIGURE 4 – Lineweaver-Burk plot for arginine deiminase activity with L-arginine as substrate. Each point represents the average for duplicate samples. Bars, range of values. The values of K_m and V_{max} were calculated to be 0.2 mM and 50 units/mg protein respectively.

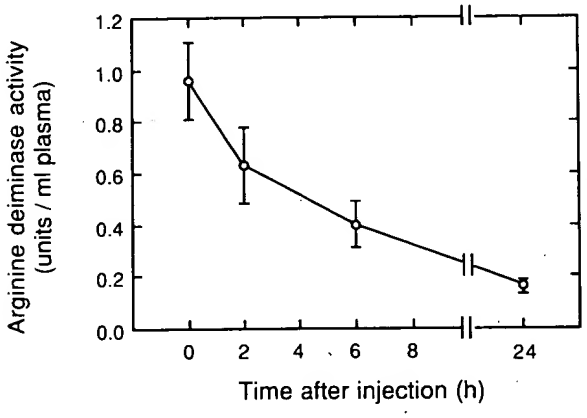

FIGURE 5 – Plasma clearance of arginine deiminase injected into mice. Three units of purified arginine deiminase was injected into the tail veins of 2 normal BDF_1 mice. Blood samples were obtained periodically from the tail vein, and the circulating activities of arginine deiminase was assayed. Each point represents the average for duplicate experiments. Bars, range of values.

In vivo *anti-tumor activity*

To assay *in vivo* anti-tumor activity, about one g of arginine deiminase was purified to homogeneity from a 100-liter culture of *M. arginini*. The 6 kinds of mouse tumor cells were injected i.p. into their syngeneic mice, and from the next day the mice received daily i.p. injections of the purified enzyme, at a dose of 0.04 mg or 0.2 mg, for 14 days. The results are summarized in Table II. At 0.2 mg/mouse, the arginine deiminase significantly retarded the growth of all tumor lines in the mouse ascites and prolonged their survival time. In the case of S-180, the arginine deiminase extended the mean survival time of the tumor-implanted mice by >66% at a dose of 0.04 mg/mouse and by >128% at a dose of 0.2 mg/mouse: of the 8 test mice, 2 and 5 mice survived for over 75 days at 0.04 mg and 0.2 mg respectively. The more prominent anti-tumor effect was observed in the mice injected with MH134 cells. When 0.2 mg of the enzyme was injected, all the test mice survived beyond the 75th day, when the test was terminated, whereas all control

TABLE I – TIME-COURSE OF PLASMA CONCENTRATION OF ARGININE AND CITRULLINE AFTER INTRAVENOUS INJECTION OF ARGININE DEIMINASE

Time after injection	Plasma concentration ± S.D. (μM) (% of control)	
	Arginine	Citrulline
Control	177.0 ± 23.3 (100)	68.4 ± 14.9 (100)
5 min	<5.0[1] (<3)	233.1 ± 31.0 (341)
3 days	<5.0[1] (<3)	289.6 ± 27.5 (423)
8 days	117.3 ± 18.7 (66)	65.8 ± 19.9 (96)

[1]The detection limit for plasma arginine level was 5.0 μM.

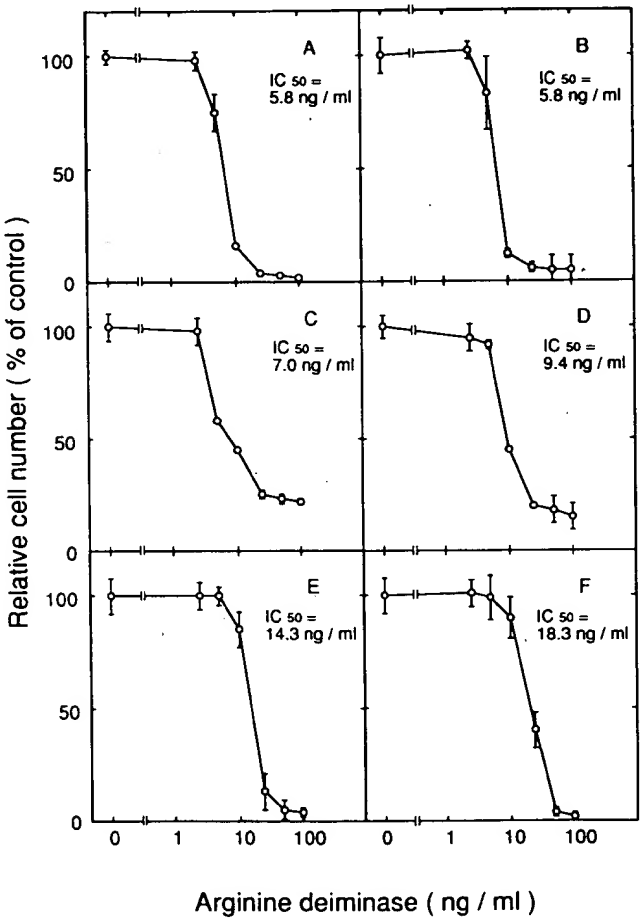

FIGURE 6 – Effect of various concentrations of arginine deiminase on growth of Meth A *(a)*, Colon 26 *(b)*, B16 *(c)*, L1210 *(d)*, S-180 *(e)* and MH134 *(f)* cells. Each point represents mean ± SD (bar) obtained from triplicate wells. The average cell number per well ($\times 10^{-4}$) in control cultures after 3 days was 44.5 (Meth A), 6.68 (Colon 26), 18.8 (B16), 40.1 (L1210), 12.8 (S-180) and 38.6 (MH134). IC_{50}, arginine deiminase concentration required for 50% growth inhibition. Other experimental conditions are described in "Material and Methods".

mice died by the 33rd day. No evidence of tumor growth and no adverse pathology related to the enzyme administration were observed when the surviving mice were subjected to necropsy. At a dose of 0.04 mg, only 1 of the 10 test mice was

TABLE II – EFFECT OF ARGININE DEIMINASE ON SURVIVAL TIMES OF TUMOR-IMPLANTED MICE

Tumor cell (number of mice)	Mean survival days ± SD (T/C %)		
	Control	0.04 mg	0.2 mg
L 1210	7.2 ± 0.4	8.2 ± 1.0	9.2 ± 0.4[2]
(n = 5)	(100)	(112)	(129)
B 16	27.1 ± 0.9	33.3 ± 5.7[1]	40.3 ± 10.6[2]
(n = 8)	(100)	(128)	(149)
Meth A	10.6 ± 1.6	10.7 ± 0.7	12.6 ± 1.7[1]
(n = 9)	(100)	(101)	(118)
Colon 26	11.1 ± 1.3	11.9 ± 1.4	21.4 ± 3.6[2]
(n = 9)	(100)	(107)	(193)
S-180	21.3 ± 9.9	> 35.3 ± 13.3[1]	> 48.4 ± 9.0[2]
(n = 8)	(100)	(> 166)	(> 228)
MH 134	25.0 ± 6.7	> 75[2]	> 75[2]
(n = 10)	(100)	(> 300)	(> 300)

Significantly different from the control value (Student's *t*-test), [1]$p < 0.05$, [2]$p < 0.01$.

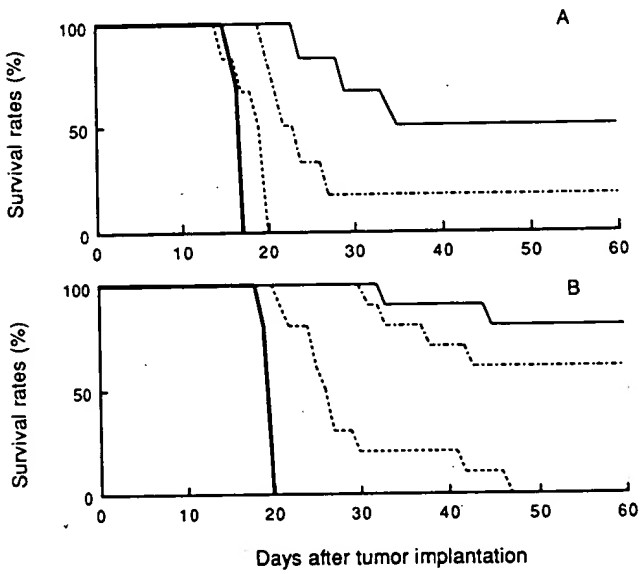

FIGURE 7 – Effect of single *(a)* and daily *(b)* intravenous injections of arginine deiminase on survival times of mice implanted with MH134 hepatoma. Mice were injected i.p. with 1×10^6 of the tumor cells on day 0, and randomly divided into control (—) and 3 test groups. The test mice had arginine deiminase injected into the tail vein. *(a)* The enzyme was once injected i.v. on day 1 at a dose of 2 (---), 10 (- · -) or 50 (—) mg/kg (n = 6); *(b)* the enzyme was injected daily i.v. from day 1 to day 10 at a dose of 0.2 (---), 1.0 (- · -) or 5.0 (—) mg/kg (n = 10). Other experimental conditions are described in "Material and Methods".

killed by tumor growth, whereas the others survived beyond 75 days without evidence of tumor growth. Among the 6 tumor lines, only Colon 26 formed solid tumors in the peritoneal cavity. The administration of arginine deiminase effectively suppressed tumor growth at a dose of 0.2 mg/mouse, suggesting that this enzyme may be effective not only for ascitic tumors but also for solid tumors.

The growth-inhibitory effect against MH134-implanted mice was also tested by i.v. administration of arginine deiminase (Fig. 7). Both a single injection and 10 daily i.v. injections significantly prolonged the survival time of the tumor-bearing mice, as did i.p. injection. The minimum effective dose was 10 mg/kg (about 0.2 mg/mouse) (Fig. 7*a*) and 0.2 mg/kg (about 0.004 mg/mouse) (Fig. 7*b*) respectively. The daily injections were judged to be superior to the single injection, because the total amount of arginine deiminase injected daily was smaller than that administered in the single injection.

Acute and sub-acute side effects of arginine deiminase were examined by injecting the purified enzyme i.v. into mice once at a dose of 1,000 mg/kg and daily at 10 mg/kg for 2 weeks. Anatomical examination revealed no apparent toxic effect in these mice. The arginine deiminase injection did not significantly change whole-body weight or the weight of major organs. Moreover, the serum levels of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were not significantly affected by the injections, indicating that arginine deiminase was not toxic to liver cells at these doses. The results showed that the side effects of arginine deiminase were very low, if any, at doses where tumor growth was effectively suppressed.

Arginine deiminase from Pseudomonas putida

Arginine deiminase was also purified from *P. putida,* essentially by the method of Shibatani (Kakimoto *et al.,* 1971; Shibatani *et al.,* 1975). The purified enzyme was composed of 2 apparently identical sub-units with M_r 45,000, as determined by SDS-PAGE. Its specific activity and K_m value were about 40 units/mg protein and 0.2 mM respectively. It showed the maximal activity at pH 6.0, but at pH 7.4 its activity decreased to 13% of the maximal activity. Most of those results were consistent with those obtained by Shibatani *et al.* (1975). When the *in vitro* growth-inhibitory activities of the arginine deiminases from *P. putida* and *M. arginini* were compared using Sarcoma 180 and MH134 as the indicator cells, IC_{50} was 10-fold higher with the former enzyme than with the latter (data not shown). It is likely that the low growth-inhibitory activity of the *P. putida* enzyme is due to its low enzymatic activity under culture conditions (pH 7.2 to 7.4).

DISCUSSION

Arginine deiminase was purified from a mass culture of *M. arginini* ATCC 23838. The purified enzyme was a dimeric protein consisting of 2 identical sub-units with Mr 45,000. Most properties of the purified enzyme, such as the molecular weight of the sub-units (45,000), pI (4.7), Km for L-arginine (0.2 mM) and stability are very similar to those of the arginine deiminase previously purified from the culture medium of a Mycoplasma-infected rat cell line (Miyazaki *et al.,* 1990). The enzyme exerted maximal enzyme activity at pH 6.0 to 7.5 and at about 50°C.

As expected from our previous finding, the purified arginine deiminase potently inhibited the *in vitro* growth of 6 kinds of mouse tumor cell lines at concentrations of 5 ng/ml or higher. Moreover, the present study first demonstrated that the enzyme could effectively retard the ascitic growth of some tumor cell lines in mice and prolong their survival time. In the cultures of Meth A, Colon 26, S-180 and MH134, almost complete cell death was attained at excess concentrations of arginine deiminase. These tumor lines, except Meth A, were also very sensitive *in vivo* to the enzyme treatment, indicating a correlation between the *in vitro* and *in vivo* assays. The *in vivo* growth of the hepatoma cell line MH134, in particular, was completely blocked by the i.p. or i.v. injection of a relatively small amount of arginine deiminase. It is interesting to note our previous finding that among 11 kinds of human tumor cell lines, a hepatoma cell line HLE was the most sensitive to the addition of arginine deiminase into culture medium (Miyazaki *et al.,* 1990). It appears possible that the tumor lines which did not show complete cell death *in vitro,* such as B16 and L1210, might contain a cell population resistant to arginine deficiency. Such resistant cells may have a high arginine-regenerating

activity by argininosuccinate synthetase and argininosuccinate lyase as compared with arginine deiminase-sensitive cells.

The chemotherapeutic effectiveness of amino-acid-degrading enzymes depends on some enzymatic properties such as the Michaelis constant (K_m), specific activity, optimum pH and stability, as well as the susceptibility of target tumor cells to lack of specific amino acids. For example, human liver arginase has a K_m value for L-arginine of 10.5 mM. The K_m value seems too high to exert enough enzyme activity in human blood, in which the normal L-arginine level is about 0.1 mM. Indeed, its *in vivo* growth-inhibitory activity is very weak or non-existent (Bach and Swaine, 1965; Savoca *et al.*, 1984). On the other hand, we also found that the arginine deiminase purified from *P. putida* has a very poor growth-inhibitory activity *in vitro* compared with the *M. arginini*-derived enzyme. The poor inhibitory activity is apparently due to its low optimum pH: it exerted negligible enzyme activity at pH 7.4. In contrast to these enzymes, the *M. arginini*-derived arginine deiminase had an appreciably low K_m and sufficient specific activity, and was active and stable at neutral pH. In addition to these properties, the blood-circulation life of the arginine deiminase injected into mice also seems to be satisfactory. It is known that L-asparaginase derived from *E. coli* has a half-life of 2.5 to 7.3 hr in mouse blood, whereas one derived from yeast is completely eliminated from blood within one hr (Wriston and Yellin, 1973). The former enzyme shows anti-tumor activity *in vivo,* but not the latter one. The half-life of the *M. arginini*-derived arginine deiminase, 4 hr, is comparable to that of the *E. coli* L-asparaginase. We also showed that the toxic effect of the arginine deiminase was not evident in mice up to doses about 100 times higher than its minimum effective dose. We have obtained data indicating that non-transformed rat liver epithelial cells (BRL) are very sensitive to arginine deficiency in sparse, growing cultures, whereas they are extremely resistant to the deficiency in confluent, quiescent cultures (data not shown). This may be a main reason why arginine deiminase has unexpectedly low side effects *in vivo.* All of the above mentioned characteristics of the *M. arginini*-derived arginine deiminase suggest that this enzyme is a promising candidate for use as a new anti-tumor drug.

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3) Takaku H, 1993, Japanese J Cancer Research, 84(11): 1195-200?
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Jpn. J. Cancer Res. 84, 1195–1200, November 1993

Chemical Modification by Polyethylene Glycol of the Anti-tumor Enzyme Arginine Deiminase from *Mycoplasma arginini*

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Amino acid-degrading enzymes are known to inhibit the growth of tumor cells in culture by depleting amino acids in the medium. Here we demonstrate that arginine deiminase (EC 3.5.3.6) from *Mycoplasma arginini* had stronger growth-inhibitory activity against all 4 kinds of tumor cell lines tested than L-asparaginase and arginase, which are well-known anti-tumor enzymes. Next, chemical modification of the arginine deiminase molecule with polyethylene glycol was shown to enhance its potency as an anti-tumor enzyme. The percentage of modified amino groups per molecule was estimated to be 51% of the total amino groups, and the average molecular weight was estimated to be about 400,000 by gel-filtration HPLC. The enzymic activity of the modified enzyme was 25.5 units/mg protein, which was equivalent to 57% of that of the native enzyme. The modified enzyme strongly inhibited growth of a mouse hepatoma cell line, MH134, at a concentration of more than 10 ng/ml, showing almost the same dose-response curve as the native enzyme. When a bolus of 5 units of the modified enzyme was intravenously injected into male BDF_1 mice, L-arginine in the blood completely disappeared within 5 min, and remained undetectable for more than 8 days. On the other hand, in the case of bolus injection of the same number of units of native enzyme, the plasma L-arginine level recovered up to 66% of the control level at 8 days. These results suggest that this modified enzyme has a longer plasma clearance time and may be more effective as a new anti-tumor agent than the native enzyme.

Key words: Arginine deiminase — Chemical modification — Polyethylene glycol — Growth inhibition — Anti-tumor agent

Potency of anti-tumor activities of amino acid-degrading enzymes depends on various enzymic properties such as the Michaelis constant (K_m), specific activity, optimum pH, and stability, as well as susceptibility of target tumor cells to the lack of specific amino acids. For example, L-asparaginase (EC 3.5.1.1),[1,2] arginase (EC 3.5.1.1),[3,4] phenylalanine ammonialyase (EC 4.3.1.5),[5] and tryptophanase (EC 4.1.99.1)[6] are known to inhibit growth of tumor cells by depleting the respective amino acids in culture. However, only L-asparaginase from *Escherichia coli* has been successfully used for clinical tumor therapy, and its use has been limited to leukemia and lymphosarcoma.[7] Recently, we reported that arginine deiminase (EC 3.5.3.6) purified from *Mycoplasma arginini* strongly inhibited the growth of various kinds of human tumor cells *in vitro*,[8] and prolonged the survival time of mice implanted with four kinds of tumors,[9] hepatoma (MH134), colon carcinoma (Colon 26), sarcoma (S-180), and melanoma (B16). No toxic effect of the enzyme was evident in the mice at doses up to about 100 times higher than the minimum effective dose *in vivo*.[9] These characteristics of *M. arginini*-derived arginine deiminase mean that it is a promising candidate for use as a new anti-tumor agent.

There are two general problems involved in the therapeutic use of such microbial enzymes, that is, antigenicity and rapid plasma clearance. To overcome these problems, many attempts have been made to alter the properties of the enzymes by chemical modification with various kinds of polymers such as polysaccharides,[10] polyethers,[11] and amino acid polymers.[12] However, the retention of catalytic activity following modification has been poor in many cases. Polyethylene glycol (PEG) is well known to have convenient properties as a chemical modifier, such as low toxicity, low antigenicity, and increased bioavailability of the modified protein.[13] So modification of enzymes, hormones, lymphokines, and other proteins with PEG has been carried out in many laboratories. For example, the modification of L-asparaginase with PEG decreased antigenicity[14] and plasma clearance rate[15,16] of the native enzyme, and enhanced its anti-tumor potency in clinical therapy.[17]

In the present study, four kinds of mouse tumor cell lines were used to compare the *in vitro* growth-inhibitory activity of arginine deiminase from *M. arginini* with that of L-asparaginase and arginase. Next, we synthesized and purified PEG-modified arginine deiminase, and defined

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its physical and enzymic properties. Moreover, to test the possible usefulness of this modified enzyme as a new anti-tumor drug, its *in vitro* growth-inhibitory activity against tumor cells and the L-arginine level in the blood after its i.v. injection into mice were examined.

MATERIALS AND METHODS

Mouse tumor cell lines Hepatoma cell line MH134 was a kind gift from Dr. H. Taguchi, SRL Company, Tokyo; colon carcinoma cell line Colon 26 was generously donated by Dr. T. Tashiro, Japanese Foundation for Cancer Research, Tokyo; and sarcoma cell line S-180 and leukemia cell line L1210 were purchased from Dainippon Seiyaku, Tokyo.

Materials Monomethoxypolyethylene glycol with average molecular weight of 5,000 (Aldrich) was used without further purification. Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) was obtained from Wako Pure Chemical Industries (Osaka). Activated PEG_2 (2-methoxy-polyethylene glycol-4,6-dichloro-1,3,5-triazine) was prepared as described by Kamisaki *et al.*[15] Arginine deiminase was purified to homogeneity from the cell extract of *M. arginini* as previously reported.[9] L-Asparaginase from *E. coli* (more than 200 units/mg protein) and arginase from bovine liver (215 units/mg protein) were purchased from Seikagaku Corporation (Tokyo) and Sigma Chemical Corporation (St. Louis, MO), respectively.

Preparation of PEG-arginine deiminase The chemical modification of arginine deiminase with activated PEG_2 was carried out by the method of Abuchowski *et al.*[11] Activated PEG_2 (1.6 mg) was added to 20 ml of enzyme solution (2.5 mg/ml) in 0.1 *M* sodium carbonate buffer (pH 9.0), and the mixture was stirred at 37°C for 30 min. Then 180 ml of cold 1 *M* potassium phosphate buffer (pH 7.0) was added to the sample solution to stop the reaction. The reaction mixture was concentrated to 30 ml with an ultrafiltration membrane (Amicon Diaflo YM-30). Next, the PEG-arginine deiminase was purified by gel-filtration chromatography on a Sephacryl S-300 HR (Pharmacia) column (4.4×90 cm) with 10 m*M* potassium phosphate buffer (pH 7.0) used for elution.

Characterization of PEG-arginine deiminase The degree of modification at amino groups in the arginine deiminase molecule was determined by measuring the amount of free amino groups by the calorimetric method using trinitrobenzene sulfonate.[18] The protein concentration of native- and PEG-arginine deminase was determined by the Lowry method with bovine serum albumin as the standard. The enzymic activity of arginine deiminase was assayed as previously described according to the method of Oginski.[19] One unit of enzymic activity was defined as the amount of enzyme that converted 1 μmol of L-arginine to L-citrulline per minute under the assay conditions employed. The molecular weight of the enzymes was analyzed by gel-filtration HPLC on a TSKgel G3000 SW_{XL} column (Toso, Tokyo).

Assay of *in vitro* growth-inhibitory activity The growth-inhibitory activities of three amino acid-degrading enzymes, arginine deiminase, L-asparaginase, and arginase, were assessed in 4 kinds of mouse tumor cell lines. The test cells (1.0×10^4 cells/well) were inoculated in triplicate into the wells of 24-well microplates, each well containing 1.0 ml of basal medium (MEM medium was used for S-180, Colon 26, and L1210 cells; and PRMI 1640 medium, for MH134 cells) supplemented with 10% fetal bovine serum (Irvine Scientific, Santana, CA). Twenty microliters of sterilized enzyme solution was then added to each well to a final concentration of 1 to 1000 ng/ml, and the plates were incubated at 37°C in a humidified 5% CO_2-95% air atmosphere. After 3 days in culture, the cell number was determined with an automatic cell counter (Coulter, Hialeah, FL). MH134 cells were used for the comparison of growth-inhibitory activities between the native enzyme and PEG-arginine deiminase at final enzyme concentrations of 2.5 to 40 ng/ml.

Analyses of amino acids in plasma The same number of units (5 units/mouse) of native enzyme or PEG-arginine deiminase was singly injected into the tail vein of male BDF_1 mice (N=3 for each enzyme). Blood samples were obtained periodically from the tail vein, and amino acids in the blood were modified by use of the fluorescent reagent NBD-F (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) according to the method of Imai and Watanabe.[20] Modified L-arginine and L-citrulline were analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a μ-Bondapack C_{18} (Waters, Milford, MA) column (10 μm, 3.9×300 mm).

RESULTS

***In vitro* growth-inhibitory activities of amino acid-degrading enzymes** The *in vitro* growth-inhibitory activities of arginine deiminase, L-asparaginase, and arginase against 4 kinds of mouse tumor cell lines are shown in Fig. 1. Among the three enzymes, arginine deiminase most strongly inhibited the growth of all tumor cells tested at concentrations of 10 or 100 ng/ml. When the enzyme was added to the culture medium at a concentration of 100 ng/ml or higher, all tumor cells were killed during the 3-day culture by the depletion of L-arginine owing to the catalytic reaction of the enzyme. The potency of growth-inhibitory activity of L-asparaginase on L1210 cells was almost the same as that of arginine deiminase. However, L-asparaginase showed about 10-fold weaker growth-inhibitory activity than arginine deiminase against the other tumor cell lines. In the case of arginase,

the growth of MH134 cells was not inhibited until a concentration of 1 μg/ml was used, and growth of the other cell lines was hardly affected even at this concentration. These results demonstrate that the *in vitro* growth-inhibitory activity of arginase was more than 100 times weaker than that of arginine deiminase against these tumor cell lines.

Preparation and properties of PEG-arginine deiminase

The native arginine deiminase was modified with monomethoxypolyethylene glycol by use of cyanuric chloride as a coupler. The synthesized PEG-arginine deiminase was purified by gel-filtration chromatography on a Sephacryl S-300 column. The eluted fractions corresponding to an apparent molecular weight of more than about 200,000 were pooled as the PEG-arginine deiminase fraction (data not shown). The purity and molecular weight of both native and modified enzymes were analyzed by gel-filtration HPLC on a TSKgel G3000 SW_{XL} column. The native enzyme was eluted at a retention time corresponding to a molecular weight of 90,000 and showed a single sharp peak (Fig. 2A). On the other hand, the modified enzyme showed a broad peak corresponding to a molecular weight range of 300,000 to 500,000 (Fig. 2B). The average molecular weight of PEG-arginine deiminase was estimated to be about 400,000. The enzyme activities of native and modified arginine deiminase were 44.5 and 25.5 units/mg protein, respectively;

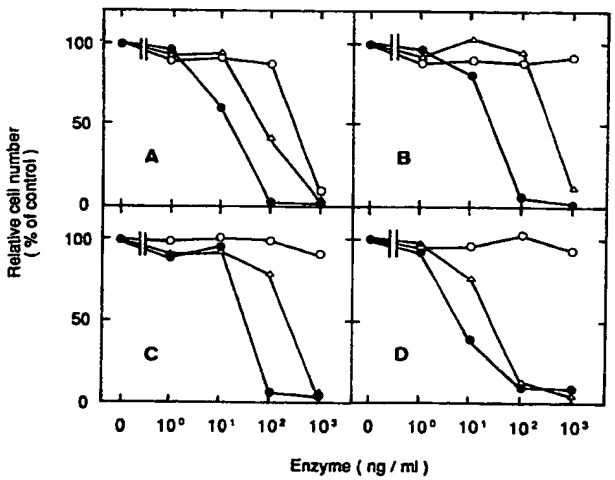

Fig. 1. Effects of arginine deiminase (●), arginase (○), and L-asparaginase (△) on growth of MH134 (A), S-180 (B), Colon 26 (C), and L1210 (D) cells. Each point represents the mean obtained from triplicate wells. The average cell number per well ($\times 10^{-5}$) in control cultures after 3 days was 13.0 (MH134), 4.97 (S-180), 8.47 (Colon 26), and 22.6 (L1210). Other experimental conditions are described "Materials and Methods."

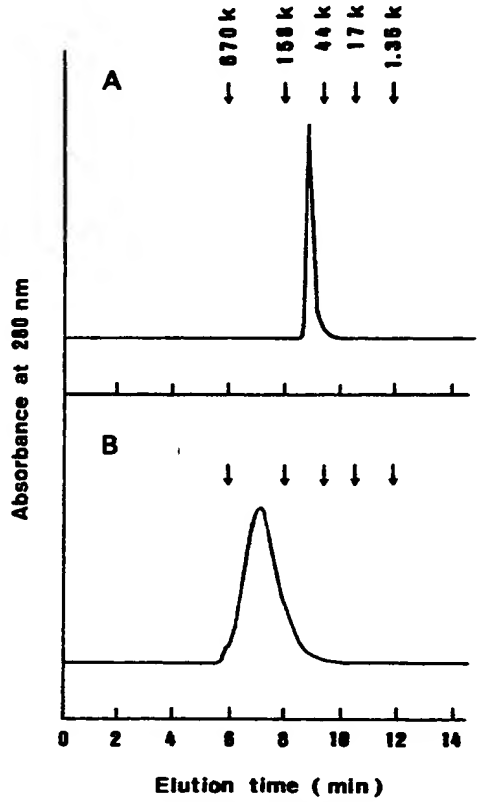

Fig. 2. Gel-filtration HPLC of native- (A) and PEG-arginine deiminase (B) on a TSK G3000 SW_{XL} column. The protein standards used were thyroglobulin (Mr 670,000), gamma globulin (Mr 158,000), ovalbumin (Mr 44,000), myoglobin (Mr 17,000), and vitamin B12 (Mr 1,350).

Table I. Molecular Weight, Enzyme Activity, and Degree of PEG-modification of Native Enzyme and PEG-arginine Deiminase

Enzyme	Molecular weight	Enzymic activity (U/mg protein)	PEG-modification (%)
Native arginine deiminase	90,000	44.5 (100%)	0
PEG-arginine deiminase	400,000	25.5 (57.3%)	51

i.e., 43% of the enzymic activity was lost by the modification. The degree of modification of the amino groups per molecule was determined to be 51% by measuring the amount of free amino groups in both native and modified enzyme molecules (Table I).

***In vitro* growth-inhibitory activity** The *in vitro* growth-inhibitory activity of native enzyme and PEG-arginine deiminase against mouse hepatoma cell line MH134 is shown in Fig. 3. Both enzymes strongly inhibited the growth of the tumor cells, showing almost the same sigmoidal dose-response curves. The concentration required for 50% growth inhibition (IC_{50}) was estimated to be about 8 ng protein/ml for the native enzyme and about 9 ng protein/ml for the modified one. When the enzymes were added to the culture medium at concentrations of more than 20 ng/ml, the tumor cells were almost completely killed during the 3-day incubation. In another *in vitro* experiment, mouse colon carcinoma cell line Colon 26 was also inhibited by these enzymes with almost the same dose-response curves as for MH134 cells (data not shown).

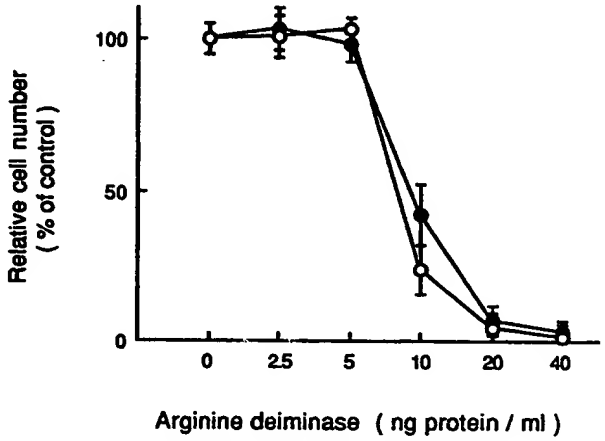

Fig. 3. Effects of various concentrations of native enzyme (○), and PEG-arginine deiminase (●) on growth of MH134 cells. Each point represents the mean ± SD (bar) from triplicate wells. The average cell number per well in control cultures after 3 days was 8.93×10^5.

Analyses of L-arginine and L-citrulline in plasma To study the potency of PEG-arginine deiminase as an anti-tumor enzyme, the concentration of L-arginine and L-citrulline in mouse plasma was analyzed periodically after a single i.v. injection of native or modified enzyme (Table II). L-Arginine was completely cleared from the plasma within 5 min after injection of either enzyme, with a reciprocal increase in L-citrulline. In the case of the modified enzyme, L-arginine remained undetectable for at least 8 days, and then recovered to 15% of the control level by 15 days, while L-citrulline was increased 16-fold over the control level at 3 days, and the level decreased gradually to 142% of the control level by 15 days. In the case of the native enzyme, L-arginine remained undetectable for 3 days and then its level recovered to 66% of the control level by 8 days. The maximum citrulline level was about 4 times that of the control at 3 days. These results indicate that the plasma clearance time of native arginine deiminase was prolonged by the modification with PEG. No toxic effect of PEG-arginine deiminase was observed in any mouse during the experiments (data not shown).

Table II. Time Course of Plasma Levels of L-Arginine and L-Citrulline in Mice after Intravenous Injection (5 units/mouse) of Native- or PEG-arginine Deiminase

Time after injection	Plasma concentration ± SD (μM) (% of control)			
	Native arginine deiminase		PEG-arginine deiminase	
	L-Arginine	L-Citrulline	L-Arginine	L-Citrulline
Control	177.0 ± 23.3 (100)	68.4 ± 14.9 (100)	242.0 ± 31.2 (100)	77.6 ± 2.0 (100)
5 min	< 5.0[a] (< 3)	233.1 ± 31.0 (341)	< 5.0[a] (< 3)	287.0 ± 34.7 (370)
3 days	< 5.0[a] (< 3)	289.6 ± 27.5 (423)	< 5.0[a] (< 3)	1245.9 ± 318.9 (1606)
8 days	117.3 ± 18.7 (66)	65.8 ± 19.9 (96)	< 5.0[a] (< 3)	340.6 ± 79.2 (439)
15 days	91.4 ± 5.4 (52)	135.5 ± 2.7 (198)	36.8 ± 40.3 (15)	109.9 ± 13.2 (142)

a) The detection limit for plasma L-arginine level was 5.0 μM.

DISCUSSION

Recently we studied the growth-inhibitory activity of arginine deiminases derived from various kinds of microorganisms, such as *Mycoplasma*, *Pseudomonas*, and *Streptococcus*. As a result, arginine deiminase purified from *M. arginini* showed potent anti-tumor activities against various kinds of tumor cells *in vitro* and *in vivo*,[8,9] whereas that from *M. hominis* showed low growth-inhibitory activity, presumably because of its acidic optimum pH (data not shown). In the present study, the *in vitro* growth-inhibitory activity of arginine deiminase from *M. arginini* was compared with that of L-asparaginase from *E. coli* and with that of arginase from bovine liver, both of which are well-known anti-tumor amino acid-degrading enzymes. Among the three enzymes, arginine deiminase most strongly inhibited the growth of all four cell lines tested. Both L-asparaginase and arginase are known to inhibit the growth of leukemia cells.[1,4] In accord with these previous findings, the former enzyme showed potent growth-inhibitory activity against leukemia cell line L1210 at a concentration of more than 10 ng/ml. However, the latter enzyme did not inhibit the growth of L1210 cells even at 1 μg/ml, whereas arginine deiminase inhibited it at 10 ng/ml. This great difference between two arginine-degrading enzymes in their growth-inhibitory activities seems to originate largely from the difference in their K_m values for L-arginine: 0.2 m*M* with arginine deiminase[9] and 10.5 m*M* with arginase.[21] These results suggest that arginine deiminase from *M. arginini* is a promising candidate as an anti-tumor drug, so we selected this enzyme as a new target protein for chemical modification.

Tumor therapy using amino acid-degrading enzymes is dependent on the plasma concentration of the amino acid that is the substrate for the enzyme, so plasma clearance time of the enzyme is a very important factor determining the efficacy of an enzyme as an anti-tumor drug. For example, it is known that L-asparaginase from *E. coli*, which has a half-life time of 2.5–7.3 h in mouse blood, shows anti-tumor activity *in vivo*, whereas the enzyme from yeast, which is completely eliminated from the blood within 1 h, does not.[22] In a previous study, we reported that arginine deiminase purified from *M. arginini* had a half-life time of 4 h, and showed anti-tumor activity in mice implanted with various kinds of tumor cells.[9] However, the *in vivo* minimum effective dose of this enzyme (10 mg/kg for single i.v. injection, and 0.2 mg/kg for 10 daily i.v. injections against mouse bearing hepatoma cell line MH134) as a biological anti-tumor reagent seems a little higher. These previous results suggest that if the stability of this enzyme in the blood stream can be increased, the potency of its anti-tumor activity would be enhanced.

In the present study, we prepared PEG-arginine deiminase with almost the same *in vitro* growth-inhibitory activity as the native enzyme (IC_{50} values were 8 ng protein/ml for native enzyme and 9 ng protein/ml for modified enzyme), whereas the enzymic activity of PEG-arginine deiminase was significantly lower than that of native enzyme (57.3%). These results suggest that PEG-arginine deiminase in the culture medium may be more stable than the native enzyme to proteases present in the fetal bovine serum added to the culture medium.

We previously reported that arginine deiminase from *M. arginini* is a dimeric protein consisting of two identical subunits.[9] Recently, our laboratory determined its complete nucleotide sequence of 1,230 bp, coding 410 amino acids (nucleotide sequence accession number in the EMBL Data Library is X54141). One subunit of this enzyme has 29 primary amino groups, i.e., 28 lysine side chains and one N-terminal residue, at which modification with activated PEG_2 is most likely to occur. Therefore when 51% of the primary amino groups in the dimeric form of this enzyme was modified by activated PEG_2, the number of covalently bound activated PEG_2 molecules (average molecular weight about 10,000) was calculated at about 30. Consequently the molecular weight of PEG-arginine deiminase was calculated at about 390,000, because the molecular weights of native enzyme and 30 equivalents of activated PEG_2 were 90,000 and 300,000, respectively. Actually the average molecular weight of modified enzyme was estimated to be about 400,000, ranging from 300,000 to 500,000, by gel-filtration HPLC in this study. This experimental result is in good accord with the calculated value.

The anti-tumor potency of arginine deiminase depends on the disappearance time of L-arginine from the blood after its administration. So we examined the L-arginine level in mouse plasma after a single i.v. injection of native enzyme or PEG-arginine deiminase. The modified enzyme significantly prolonged the L-arginine disappearance time in mouse plasma as compared with the native enzyme. When a single i.v. injection of 5 units (about 0.2 mg protein) of PEG-arginine deiminase was administered to mice, L-arginine remained undetectable in the blood stream for at least 8 days. This result suggests that an injection of PEG-arginine deiminase at a dose of 5 units/mouse (about 10 mg protein/kg) only once a week may be sufficient to exhibit maximum *in vivo* anti-tumor activity, whereas 10 daily injections of native enzyme at a dose of 5 units/mouse (about 5 mg protein/kg) were needed to achieve the same result in the previous study.[9] However, further therapeutic, toxicological, and immunological studies on PEG-arginine deiminase are required prior to its clinical trial as a new anti-tumor agent.

(Received June 14, 1993/Accepted August 16, 1993)

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